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Award Number: W81XWH-09-1-0297

TITLE: Identification of Estrogen Receptor Beta Binding Sites in The Human Genomes

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REPORT DATE: April 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2012	Annual Summary	1 April 2009 – 31 March 2012

4. TITLE AND SUBTITLE

Identification of Estrogen Receptor Beta Binding Sites in The Human Genomes

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0297

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Chicago
Chicago, IL 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

ERβ knock-out mice developed prostatic hyperplasia at late age, suggesting an important role of ERβ in the development of the prostate as well as prostate cancer. Here we describe a study that thoroughly investigates the genomic function of ERβ. A FLAG-tagged ERβ was stably expressed in MCF7 C4-12 cells, which allowed ERβ transcriptional activity to be studied in an ERα-independent background. In this cell model, we identified 3166 ERβ genomic binding sites. Although different from ERα genomic binding sites, these ERβ genomic binding sites had similar global distribution pattern and enriched transcription factor binding motifs to those associated with ERα genomic landscape. In conjunction with nascent RNA profiling at the time of ERβ binding events, ERβ target genes were found to be enriched in apoptotic and developmental processes. This was in agreement with our result showing that ERβ suppressed cell growth in response to E2 treatment. Interestingly, 30% of ERβ binding sites carried EBF1 (Early B-cell Factor 1) binding motif. The interaction between EBF1 and ERβ correlated with down-regulated ERβ protein stability and downstream activity. Similar phenomenon was observed between EBF1 and ERα. These results, at least to our knowlecge, were the first to indicate crosstalk between EBF1 and the estrogen receptors on a large scale.

15. SUBJECT TERMS
Genome-wide mapping; estrogen receptor beta

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Beside being the major target of androgens, the prostate is also influenced by estrogens. In the adult male, 17-beta-estradiol (E2), classically considered the female sex hormone, is mainly produced by adipose tissue, adrenal glands, testicles and the prostate[1]. On the cellular and molecular level, E2 mainly exerts its effect via the two estrogen receptors, ERα and ERβ. ERα , as a marker for breast cancer, has been heavily studied, leading to a myriad of improvement in breast cancer treatment and prevention[2]. On the other hand, the functions of ERβ remain largely elusive if not controversial. Interestingly, ERβ knock-out mice were reported to develop prostatic hyperplasia at late age[3]. Moreover, different lines of studies have suggested that ERβ played an important role in anti-proliferation[4], immunoprotection[5], and detoxification in the prostate[6]. Therefore, ERβ strikes as an important component in prostatic normal development as well as tumorigenesis. Because ERβ regulates gene expression mainly at the transcription level, here ERβ genome-wide binding sites were mapped in an ERα -negative cell model using ChIP-seq technology. In conjunction with nascent RNA profiling at the time of binding events, detected by GRO-seq technology, our results provided a better knowledge of ERβ target genes as well as how ERβ regulates their expressions on the global scale. Ultimately, this will give rise to a better model to study this nuclear receptor.

BODY

Aim1 – To identify genome-wide ERβ binding regions

The lack of a reliable antibody for ERβ led to many controversies surrounding the presence and functions of this steroid receptor[8]. Due to the same reason, detecting ERβ binding sites, via chromatin immunoprecipitation (ChIP) assays, was also delayed. Therefore, we proposed to generate cancer cell lines stably expressing ERβ as a Flag-tagged fusion protein (Flag-ERβ). Since specific and reliable antibodies raised against the Flag were broadly available, the fusion protein would be efficiently immunoprecipitated in ChIP conditions.

In order to map ERβ genome-wide binding sites in a non-biased manner, we chose ChIP followed by massive parallel sequencing (ChIP-seq) as our main approach. This technology was widely proven to produce reliable data with higher resolution compared to the conventional ChIP-chip assay (ChIP followed by tiling microarray).

Also, in order to study ERβ genomic functions without the interference of ERα , we wished to introduce the Flag-ERβ into two well-characterized prostate cancer cell lines LNCaP, PC3 (in which ERα is undetectable), and a breast cancer cell line MCF7/C4-12 (a derivative of the MCF7 cell line which does not express ERα).

C4-12/Flag.ERβ cells stably expressed Flag-tagged ERβ:

Due to a variety of reasons (low viral infection efficiency, low cell growth rates, etc.), we were not able to generate the prostate cancer cells stably expressing Flag-ERβ at first. However, the DNA construct of the fusion protein appeared to be integrated into the genome the MCF7/C4-12 cells at a much higher rate. The efficiency of viral infection could be ranked highest in MCF7/C4-12 cells, low in PC3 cells, and very low in LNCaP cells. Moreover, the MCF7-C4/12 cells grew at a very fast pace, which allowed generating material for the downstream assays much more efficiently. Therefore, we used the MCF7/C4-12 cells stably expressing Flag-ERβ (C4-12/Flag.ERβ) to optimize the condition for the ChIP-seq assay, while developing the prostate cancer cells stably expressing Flag-ERβ.

Although we were able to express Flag-ERβ in LNCaP and PC3 cells, the stable expression level of the ectopic protein was too low. Moreover, these cell lines appeared to quickly lose Flag-ERβ expression. Meanwhile, we were advancing our analyses of ERβ genomic functions using the C4-12/Flag.ERβ cells. Although this cell line derived from the breast cancer cell line MCF7, the cells had gone through significant changes so that they should no longer be considered a cell model for breast cancer. However, these cells still provided sufficient cellular environment to study the biology of ERβ. Therefore, we decided to carry on our project using the C4-12/Flag.ERβ as our cell model.

Mapping ERβ genomic binding sites

C4-12/Flag.ERβ cells were serum-starved for 24 hours before treated for 1 hour with 10nM estradiol (E2). Cells treated with the same volume of vehicle (ethanol) were used as control. The cells were crosslinked and proceeded through ChIP assay, in which anti-Flag M2 antibody (Sigma-Aldrich) was used to pull down the Flag-ERβ. The immunoprecipitated DNA libraries were prepared according to the Illumina ChIP-seq library prep kit. Samples were then submitted to high throughput sequencing with Solexa technology at the IGSB High-throughput Genome Analysis Core (Argonne National Laboratory, IL).

As we proceeded through the ChIP-seq assay with the C4-12/Flag.ERβ cells, our data confirmed the high efficiency of the optimized ChIP-seq conditions. We were also able to identify more 3166 binding sites at high stringency (1% FDR). A few binding sites were selected at random to confirm with ChIP-qPCR (Figure 1C). The global distribution of ERβ binding sites at high stringency is presented in figure 1A. Moreover, our motif analysis identified binding motifs of ERs, known ERβ interactors as well as those of novel interactors (figure 1B).

The binding motif of EBF1 (Early B-cell Factor 1) was enriched in 30% ERβ binding regions. Moreover, EBF1 mapping experiments revealed binding motifs of the estrogen receptors (Triggs, J., unpublished data). These evidence, at least to our knowledge, were the first to suggest a genomic interplay between EBF1 and ERβ. Furthermore, EBF1 was downregulated in prostate cancer cells (Oncomine). This prompted us to perform downstream assays, described below, to further investigate such crosstalk.

Crosstalk between EBF1 and ERβ

EBF1 downregulated ERβ transcriptional activity

We performed a luciferase reporter assay to assess if EBF1 affected ERβ transcriptional activity. Briefly, C4-12 cells were transiently transfected with a plasmid carrying a Firefly Luciferase gene preceded by three tandem estrogen response element (ERE), a Renilla Luciferase plasmid (to serve as transfection efficiency control), and an ERβ plasmid. These cells were also transiently transfected with a pcDNA as vector control or an EBF1 plasmid. Upon estradiol induction, ERβ was recruited to the ERE and facilitate the expression of lucierase protein, resulting in an increase of luciferase activity. However, in the presence of EBF1, this activity was significantly reduced in a ligand independent manner (Figure 2A).

Furthermore, EBF1 also downregulated transcriptional activities exerted by ERα and the two chimeric receptors in a ligand independent manner. Interestingly, constructs carrying ERβ AF1 domain (full length ERβ and ERβ/alpha) were more affected by EBF1 comparing to those carrying ERα AF1 domain (full length ERα and ERα /beta) (Figure 2A). Such results suggested that

the association between EBF1 and the estrogen receptors involved the N-terminal regions of the estrogen receptors.

EBF1 inversely correlated with ERβ protein stability

Since previous reports had been suggesting the role of the AF1 domain in the degradation of the estrogen receptors, we were interested to see if EBF1 participated in such regulation. C4-12 were transiently transfected with ERβ in the presence or absence of EBF1. After cells had been serum starved for 24hours, cell lysates were collected after 3 hours of treatment with estradiol (E2) or vehicle. Indeed, the presence of EBF1 inversely correlated with ERβ protein stability while leaving ERβ transcript level unchanged (data not shown) in a ligand independent manner (figure 2B). These results further suggested the role of EBF1 in the regulation of ERβ on the protein levels. Similar effect of EBF1 was observed on ERα protein level (Figure 2B).

EBF1 was coimmunoprecipitated with ERβ

Although we wished to test if EBF1 directly interacted with ERβ, the decreased ERβ protein level prevented such assay. In order to go about this issue, cell lysates were collected after 3hours of treatment with E2 or vehicle, in the presence of MG132, a proteasome inhibitor. In such condition, EBF1 was co-immunoprecipitated with ERβ (Figure 2C), indicating a direct interaction. EBF1 was also coimmunoprecipitated with ERα (figure 2C).

Aim 2 – To identify genes directly regulated by ERβ

Because emerging evidence had shown that trimethylation of lysine 4 on histone 3 (H3K4me3) was a reliable marker for active promoters, we first attempted to use this as the marker for actively transcribed genes with ERβ binding events, hence ERβ direct target genes. However, our analyses revealed that the hormone treatment time of 1 hour was too short for differential modifications to occur.

Alternatively, recent advances in high throughput sequencing technology have allowed the development of a new technology, namely global nuclear run-on followed by sequencing (GRO-seq). This technology provides the detection of nascent RNA on a global scale[9,10]. We have successfully applied this new technology to identify ERβ direct target genes.

GRO-seq

In order to capture gene regulation by ERβ binding onto the chromatin, cell nuclei were extracted and collected after 1 hour of E2 or vehicle treatment (the same time point of ERβ genomic binding events). Applying the GRO-seq technology, nascent RNA from these nuclei, which represented genes being actively transcribed, were extracted and processed for high throughput sequencing. The results were lists of genes being actively regulated at the time of ERβ binding events. These gene lists were overlapped with the list of genes harboring ERβ binding sites, revealing ERβ direct target genes. Among these direct targets, 209 genes were up-regulated while 133 were down-regulated.

Gene ontology analyses showed that these ERβ targets were enriched in apoptotic and developmental processes (Figure 3A). The fact that similar processes (but different genes) were found enriched in both up- and down-regulated genes suggested a switch from one pathway to another one in response to estradiol. Selected targets were chosen to confirm the assay by RT-qPCR for mRNA extracted at 2hour treatment time (Figure 3B). The presence of EBF1 also abolished the hormone responsive regulation of ERβ target genes (Figure 3C).

Aim 3 – Confirmation of ERβ target genes focusing on prostatic angiogenesis

We originally proposed to investigate the role of ERβ in regulating genes related to angiogenesis. However, according to gene ontology analyses, this process was not enriched in ERβ target genes. Therefore, we focused the confirmation of ERβ target genes on those related to apoptosis and development.

Briefly, C4-12/Flag.ERβ cells were starved in 5% charcoal-stripped serum for 24hr; these cells were then treated with E2 or Vehicle (ethanol) as negative control. After 4 days of treatment, cell confluency, measured by the Incucyte system (Essen BioScience), was used to determine cell proliferation. As shown in figure 4, E2 significantly suppressed C4-12/Flag.ERβ cell growth. Interestingly, as EBF1 was introduced to these cells via transient transfection, cell proliferation was also suppressed. Moreover, EBF1 appeared to dominate ERβ, release these cells from hormone reponsiveness due to ERβ (figure 4).

KEY RESEARCH ACCOMPLISHMENTS

- Identified a novel crosstalk between EBF1 and the estrogen receptors.

REPORTABLE OUTCOMES
N/A

SUMMARY

Using the C4-12/Flag.ERβ cell line that we generated, we were able to map ERβ genomic binding regions in an ERα -independent background. While most ERβ binding regions were different than ERα binding sites, they share similar global distribution patterns and enriched transcription factor binding motifs. In order to assess the functionalities of ERβ binding sites, we are applying the GRO-seq technology to profile nascent RNA being generated at time of ERβ binding events. Gene ontology analyses showed that ERβ target genes were enriched in apoptotic and developmental processes. This was in agreement with our cell proliferation assay as well as the hypothesis that ERβ had anti-proliferative effect. Our extensive analyses of ERβ binding sites also revealed an interesting crosstalk between ERβ and EBF1, a crucial transcription factor regulating the process of B-cell differentiation. Further experiments suggested a direct interaction between EBF1 and ERβ, resulting in decreasing ERβ protein stability and downstream activity in luciferase reporter assays. Similar phenomenon was observed between EBF1 and ERα. To our knowledge, this is the first to indicate crosstalk between EBF1 and the estrogen receptors.

Training progress

- Keystone symposium – Nuclear Receptors: Signaling, Gene Regulation and Cancer (2010).
- The PCRP IMPaCT 2011 Conference.
- GRO-seq workshop with Dr. Lee Kraus's research group (University of Texas Southwestern, 2011).
- ENDO 2011: The 93rd Annual Meeting and Expo.

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SUPPORTING DATA

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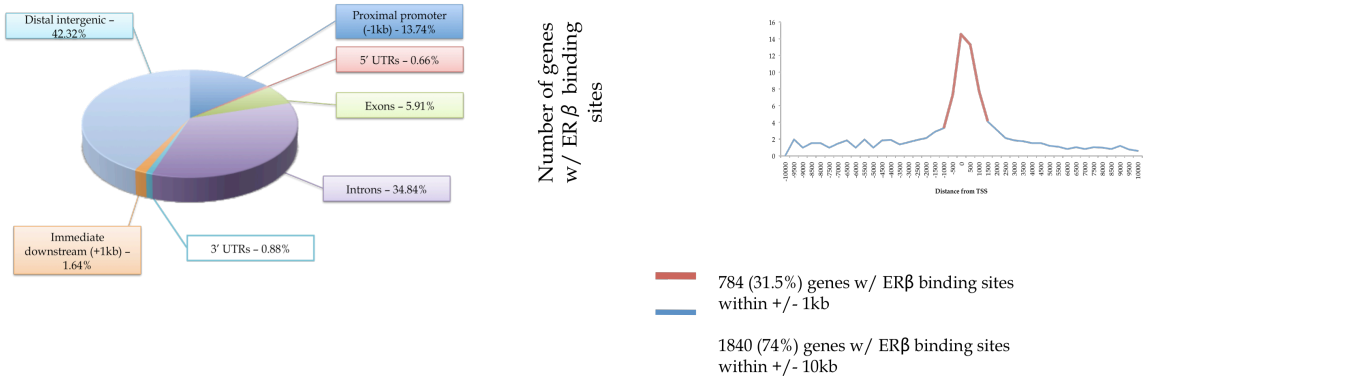

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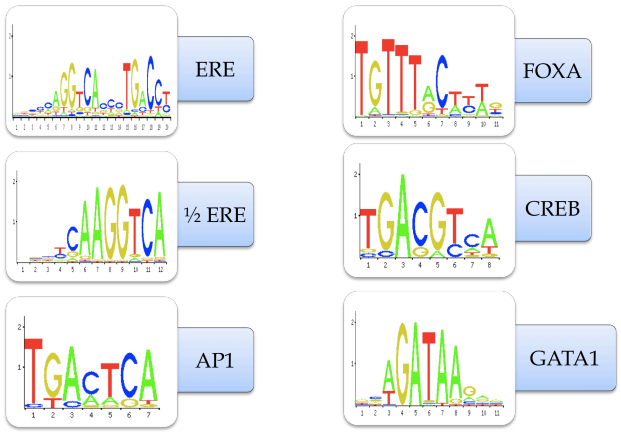

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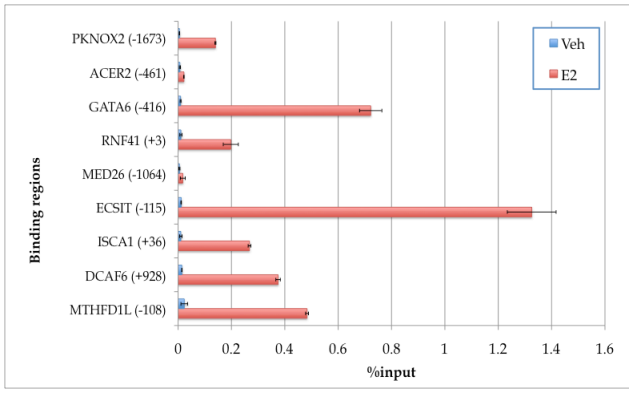
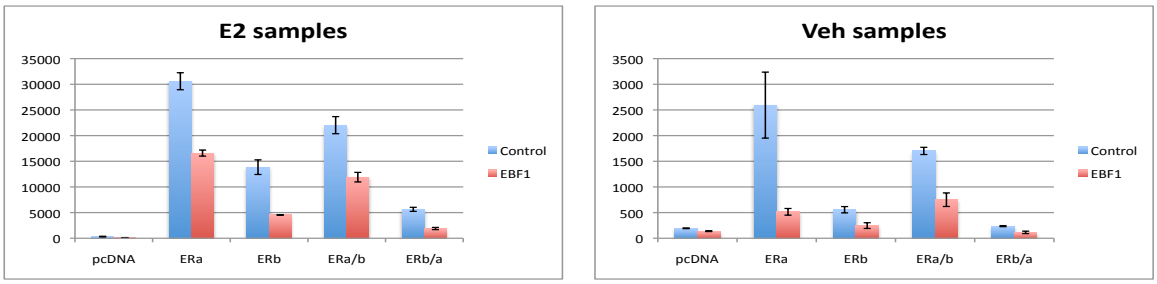


Figure 1 – Mapping ERβ binding sites in C4-12/Flag.ERβ cells.
A – Global distribution of 3166 ERβ binding regions.
B – Enriched transcription factor binding motifs found in ERβ binding sites.
C – Confirmation of randomly selected binding sites with ChIP-qPCR.

A.



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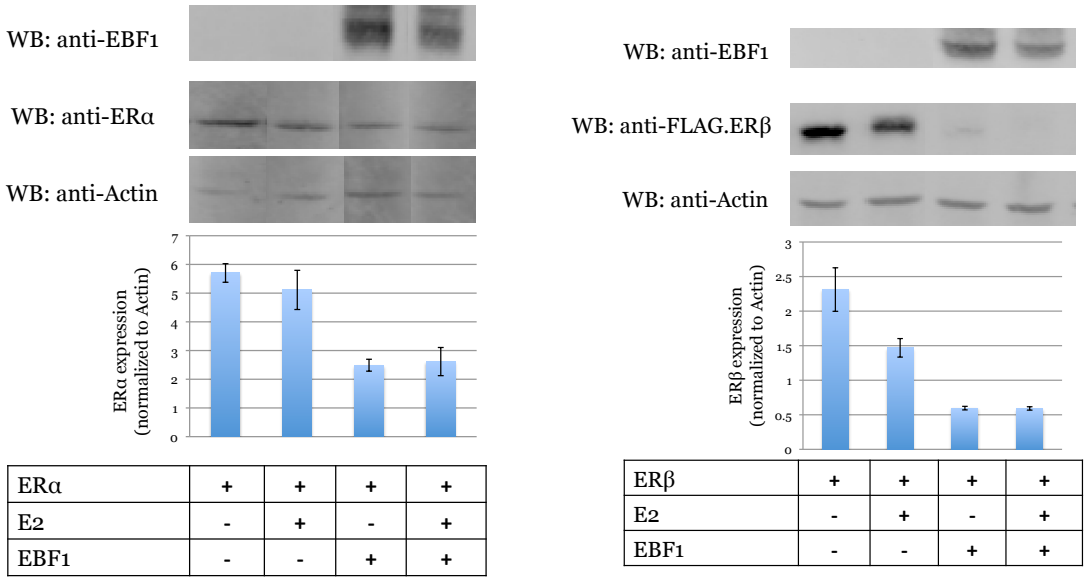


ERα	+	+	+	+
E2	-	+	-	+
EBF1	-	-	+	+

ERβ	+	+	+	+
E2	-	+	-	+
EBF1	-	-	+	+

C.

	IP: anti-FLAG.ER β		
ER β	+	+	-
EBF1	+	+	+
E2 (4hr)	-	+	-

	IP: anti-ER α		
ER α	+	+	-
EBF1	+	+	+
E2 (4hr)	-	+	-

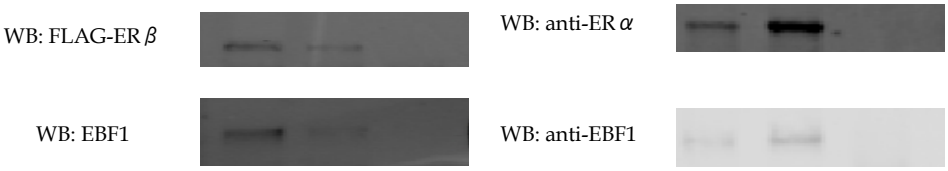


Figure 2 – Crosstalk between EBF1 and ER.
A – EBF1 suppressed ER activity in luciferase reporter assays.
B – EBF1 correlated with decrease ERα and ERβ protein stability.
C – EBF1 was coimmunoprecipitated with ERα and ERβ.

A.

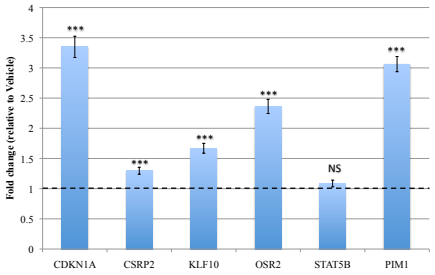
209 Up-regulated direct targets

- Differentiation
- Development
- Transcription
- Metabolic processes
- Apoptosis
- Proliferation

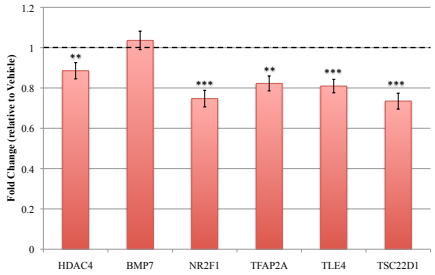
133 Down-regulated direct targets

- Transcription
- Metabolic processes
- Development
- Morphogenesis

B.



Up-regulated genes



Down-regulated genes

C.

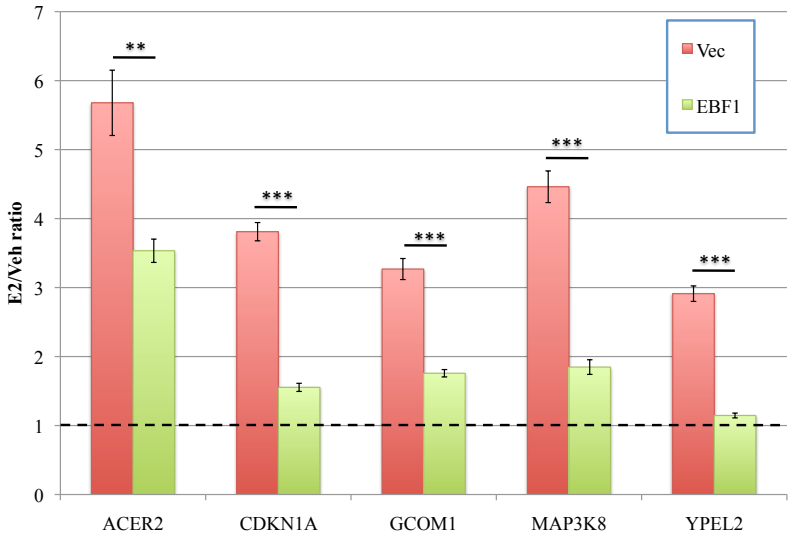


Figure 3 – ERβ target genes identified by GRO-seq
A – Gene ontology analyses revealed target genes enriched in apoptosis and development.
B – Confirmation of target genes with RT-qPCR analyses for mRNA collected at 2hr treatment time.
C – EBF1 presence abolished hormone responsive gene regulation of ER target genes.

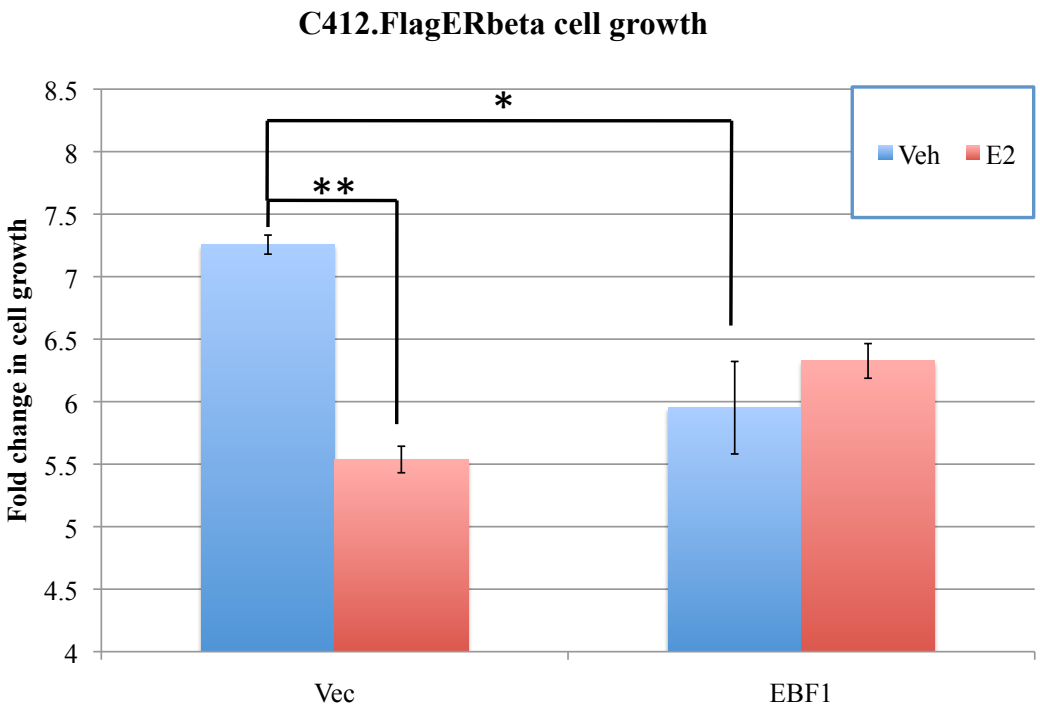


Figure 4 – C4-12/Flag.ERβ cell proliferation was suppressed by E2 and, independently, by EBF1.

APPENDICES

Abstracts for past meetings

- Thien Le, Matthew Walker, Geoffrey Greene. "Genomic functions of the estrogen receptor β" (2010) Keystone symposium – Nuclear Receptors: Signaling, Gene Regulation and Cancer.

Considerable effort by numerous laboratories has resulted in an improved understanding of estrogen and SERM action mediated by the two estrogen receptors, ERα and ERβ. However, many of the targets for ERβ in cell physiology remain elusive. Here we propose a study that thoroughly investigates the transcriptional targets and functions of ERβ.

MCF7 C4-12 cells, which do not express either ER, were engineered to stably express a FLAG-tagged ERβ. In this cell model, ERβ transcriptional activity can be studied in an ERα-independent manner. According to our ChIP-seq results (chromatin immunoprecipitation followed by deep sequencing with Solexa technology), <14% ERβ binding sites were within proximal promoter regions of putative target genes, while most binding events occurred either in intronic (34%) or distal intergenic (42%) regions. The most prevalent binding motif was the canonical ERE. Analysis of motifs within binding sites also revealed other transcription factor sites that potentially influence ERβ functions. In conjunction with active promoter, marked by H3K4me3, our results allow a global picture of ERβ transcriptional activity. In a related project, using phage display and exon microarray as a novel method for screening protein-protein interactions, we have identified novel interactions between ERβ and other coregulators. The combined results will allow the identification of ERβ target genes as well as how ERβ regulates their expressions. Ultimately, this will give rise to a better model to study this nuclear receptor.

- Thien Le, Geoffrey Greene. "Early B-cell Factor 1 regulated estrogen receptor β activity on a genomic scale" (2011) The PCRP IMPaCT 2011 Conference.

Considerable effort by numerous laboratories has resulted in an improved understanding of estrogen and SERM action mediated by the two estrogen receptors, ERα and ERβ. However, many of the targets for ERβ in cell physiology remain elusive. Interestingly, ERβ knock-out mice developed prostatic hyperplasia at late age, suggesting an important role of ERβ in the development of the prostate as well as prostate cancer. Here we describe a study that thoroughly investigates the transcriptional targets and functions of ERβ. MCF7 C4-12 cells, which do not express either ER, were engineered to stably express a FLAG-tagged ERβ. In this cell model, ERβ transcriptional activity can be studied in an ERα-independent background. According to our ChIP-seq results (chromatin immunoprecipitation followed by deep sequencing with Solexa technology), <15% ERβ binding sites were within proximal promoter regions of putative target genes. The majority of binding events occurred either in intronic (45%) or intergenic (36%) regions. While the most prevalent binding motif was the canonical ERE, ~30% of ERβ binding regions also carried the binding motif of

EBFs (Early B-cell Factor 1). This result suggests that EBF1 might potentiate or attenuate ER transcriptional activities on a global scale. In our luciferase reporter assay, EBF1 down-regulated both ERα and ERβ activities. Interestingly, ER constructs carrying the AF1 domain of ERβ (full length ERβ and ERβ/α chimera) were more affected compared to those carrying ERα AF1 domain (full length ERα and ERα/β chimera). Moreover, the ectopic expression of EBF1 correlated with reduced ERβ protein stability, which might have explained the suppressed transcriptional response in luciferase reporter assay. These results, at least to our knowledge, are the first to indicate crosstalk between EBF and ER transcriptional activities on a large scale. The roles of other proteins in the EBF family (i.e. EBF2, EBF3, EBF4) in influencing ERβ activity are under active investigation. Moreover, in conjunction with gene expression profiling, still to be completed, our results should reveal a global picture of ERβ transcriptional activity, as well as the role of EBF proteins in this process.

- Thien Le, Geoffrey Greene. "Early B-cell Factor 1 regulated estrogen receptor β activity on a genomic scale" (2011) ENDO 2011: The 93rd Annual Meeting and Expo.

Considerable effort by numerous laboratories has resulted in an improved understanding of estrogen and SERM action mediated by the two estrogen receptors, ERα and ERβ. However, many of the targets for ERβ in cell physiology remain elusive. Here we describe a study that thoroughly investigates the transcriptional targets and functions of ERβ. MCF7 C4-12 cells, which do not express either ER, were engineered to stably express a FLAG-tagged ERβ. In this cell model, ERβ transcriptional activity can be studied in an ERα-independent background. According to our ChIP-seq results (chromatin immunoprecipitation followed by deep sequencing with Solexa technology), <15% ERβ binding sites were within proximal promoter regions of putative target genes. The majority of binding events occurred either in intronic (45%) or intergenic (36%) regions. While the most prevalent binding motif was the canonical ERE, ~30% of ERβ binding regions also carried the binding motif of EBFs (Early B-cell Factor 1). This result suggests that EBF1 might potentiate or attenuate ER transcriptional activities on a global scale. V5-tagged EBF1 was co-immunoprecipitated with FLAG-tagged ERβ, demonstrating a direct interaction in a ligand independent manner. Moreover, in our luciferase reporter assay, EBF1 down-regulated both ERα and ERβ activities. Interestingly, ER constructs carrying the AF1 domain of ERβ (full length ERβ and ERβ/α chimera) were more affected compared to those carrying ERα AF1 domain (full length ERα and ERα/β chimera). These results, at least to our knowledge, are the first to indicate crosstalk between EBF and ER transcriptional activities on a large scale. Because EBF2, EBF3, and EBF4 transcripts were found to be abundant in MCF7 C4-12 cells expressing FLAG-tagged ERβ (EBF3 > EBF2/EBF4 > EBF1), the roles of these proteins in influencing ERβ activity are under active investigation. Moreover, in conjunction with gene expression profiling, still to be completed, our results should reveal a

global picture of ERβ transcriptional activity, as well as the role of EBF proteins in this process.